

June 2004

Autocrine/Paracrine Effects of Insulin-Like Growth Factor-I on Osteoblasts

Anitha Potluri

Follow this and additional works at: https://opencommons.uconn.edu/sodm_masters

Recommended Citation

Potluri, Anitha, "Autocrine/Paracrine Effects of Insulin-Like Growth Factor-I on Osteoblasts" (2004). *SoDM Masters Theses*. 106.
https://opencommons.uconn.edu/sodm_masters/106

**AUTOCRINE/PARACRINE EFFECTS OF INSULIN- LIKE GROWTH
FACTOR-I ON OSTEOBLASTS**

Anitha Potluri

B.D.S University of Gulbarga

India

A Thesis

Submitted in the Partial Fulfillment of the

Requirements for the Degree of

Master of Dental Science

At the

University of Connecticut

2004

APPROVAL PAGE

Master of Dental Science Thesis

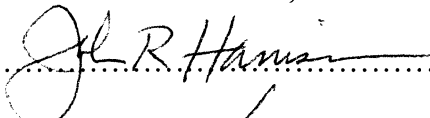
AUTOCRINE/PARACRINE EFFECTS OF IGF-I ON OSTEOBLASTS

Presented by

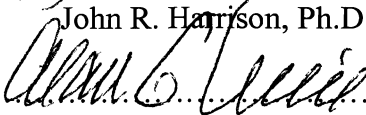
Anitha Potluri, B.D.S

Major Advisor.....

Barbara E. Kream, Ph.D.

Associate Advisor.....

John R. Harrison, Ph.D.

Associate Advisor.....

Alan G. Lurie, DMD., Ph.D.

University of Connecticut

2004

TABLE OF CONTENTS

Title	Page
Title Page	i
Approval Page	ii
Table of Contents	iii
List of Figures	iv
Introduction	1
Literature Review	2
General Objective	7
Hypothesis	7
Specific Objectives	7
Materials and Methods	8
Results	15
Discussion	19
Summary and Conclusions	23
Figures and Figure Legends	24
Literature Cited	40

LIST OF FIGURES

Figure	Page
1. Schematic diagram of the pOBCol3.6-IGF transgene and PCR genotyping of mice	25
2. Cell growth in primary calvarial osteoblast cultures	27
3. Alkaline phosphatase staining in primary osteoblast cultures	29
4. Mineralization in primary calvarial osteoblast cultures	31
5. GFP expression in primary calvarial osteoblast cultures	33
6. mRNA expression markers in primary calvarial osteoblast cultures	36
7. Expression of IGF transgene mRNA by RT-PCR analysis	39

INTRODUCTION

Insulin-like growth factor-I (IGF-I) functions as an anabolic growth factor for bone *in vivo* by stimulating preosteoblast proliferation and osteoblast differentiation. Studies in transgenic mouse models suggest the importance of paracrine/autocrine actions of IGF-I in addition to its endocrine functions. The purpose of this present study was to investigate the paracrine/autocrine effects of IGF-I on proliferation and differentiation of cells of the osteoblast lineage. Primary osteoblast cultures were obtained by serial digestion of neonatal calvariae from wild type (WT) and pOBCol3.6-IGF transgenic (TG) mice. These mice overexpress IGF-I in cells of the osteoblast lineage. Cell growth was significantly increased at day 4 in TG cultures compared to WT. AP activity was measured as an early differentiation marker on days 14 and 21. Compared to WT cells, there was a significant decrease in AP activity in TG cells on day 21. The expression of osteoblast differentiation markers including Col1a1, BSP, and OC mRNA were not significantly affected on days 7, 14 and 21. On day 21, von Kossa staining for mineralization and expression of Col2.3-GFP, a late-stage marker of osteoblast differentiation, were not altered in TG cultures. In conclusion, IGF-I overexpression driven by a 3.6-kb Col1a1 promoter fragment in primary calvarial osteoblasts transgenic mice showed an increase in osteoblast proliferation and had no significant effect on differentiation.

LITERATURE REVIEW

Bone is a specialized connective tissue that has three functions: mechanical support, protection of vital organs and maintenance of mineral homeostasis. Bone remodeling is the continuous coupled cycle of breakdown and rebuilding. Bone is composed of two major cell types: osteoblasts are bone-forming cells; osteoclasts are bone resorbing cells. Bone formation and bone resorption are coordinated as a part of turnover mechanism of bone remodeling in which resorption occurs first, followed by formation at the same site in discrete units throughout the skeleton. Osteoclasts are derived from haemopoietic stem cells in the bone marrow, specifically cells of the monocyte-macrophage lineage. Local factors are released in the bone microenvironment upon hormonal and/or mechanical signals, which activate preosteoclasts to differentiate into large multinucleated osteoclasts. Multinucleated osteoclasts attach to bone and secrete hydrogen ions and lysosomal enzymes, which resorb the bone mineral and matrix in that defined area. After bone resorption, osteoclasts probably undergo apoptosis. Macrophage-like mononuclear cells now cover the surface and form a proteoglycan-cement line, which demarcates the resorption cavity. Then, preosteoblasts proliferate and differentiate into mature osteoblasts, which originate from pluripotent mesenchymal stem cells of the bone marrow. Mesenchymal cells also carry the potential to become fibroblasts, chondrocytes, adipocytes, or muscle cells. Osteoblasts synthesize and secrete

type I collagen, alkaline phosphatase osteocalcin, osteonectin, bone sialoprotein and other matrix proteins that comprise osteoid, which eventually becomes mineralized to form bone. Other cells of the osteoblast lineage include lining cells, which are found opposed to inactive bone surfaces, and osteocytes, which have become entrapped within the bone matrix (1).

The bone formation that occurs during development and remodeling requires osteoblast proliferation and differentiation. Regulation of these biological processes involves sequential expression of cell growth and tissue specific genes, giving rise to a series of regulatory signals. Each component of regulatory cascade indicates a step in physiological control. The expression of cell cycle and cell growth regulating genes (proliferation) and genes associated with the maturation (differentiation) of the osteoblast phenotype changes as the extracellular matrix (ECM) develops mineral in normal cell cultures. These genes include histone (H4), TGF- β , fibronectin (FN), type I collagen (COL), osteopontin (OP), alkaline phosphatase (AP), matrix Gla protein (MGP), osteocalcin (OC) and collagenase (C'ASE). There are three major stages in the osteogenic developmental sequence: proliferation, matrix development and maturation and, mineralization. There are two transition points in the developmental sequence. The first is at the completion of proliferation when genes associated with matrix development and maturation are up-regulated; the second occurs at the onset of extracellular matrix mineralization. The proliferation period supports the synthesis of a type I collagen-fibronectin ECM. Expression of osteoblast phenotypic genes such as OC and BSP are

suppressed during the growth period. In the post-proliferative period, genes such as BSP and OC are activated. Mature osteoblasts and osteocytes are associated with OC expression and the mineralized nodules (2).

Bone remodeling depends on many factors. The insulin-like growth factors (IGFs) are important anabolic effectors during bone remodeling. IGFs are 7-kDa proteins that shares structural homology with proinsulin (3). IGFs can act in an autocrine/paracrine fashion to regulate bone cell differentiation and function. There are two known IGF peptides: IGF-I and IGF-II. The actions of IGFs on bone metabolism are modulated by multiple regulatory components of the IGF system, including IGF-I and IGF-II, type I and type II IGF receptors, at least six high-affinity IGF-binding proteins (IGFBP-1 to -6), and IGFBP proteases. The mature IGF-I peptide has four contiguous domains termed B, C, A, and D, which are highly conserved among mammals. In vitro, IGFs increase AP activity, a marker of differentiation (4). IGF-I stimulates collagen synthesis and decreases collagen degradation, which is consistent with the inhibitory effect of IGF-I on interstitial C'ASE expression in rat osteoblast cultures (5). IGF-I also inhibits osteoblast apoptosis (6) (7).

In vivo, IGF-I was originally proposed to act in an endocrine manner to mediate the actions of growth hormone (GH), which was coined the “somatomedin hypothesis” (8). This hypothesis states that somatic growth is regulated by GH, which primarily acts on liver to stimulate IGF-I production. IGF-I then enters the circulation, reaches its target tissues and regulates cell function. The critical role of the IGF system has been

established in mice by genetic ablation of genes for IGF-I and IGF-II and their receptors. *Igf1*^{-/-} mice are 40% smaller than wild type littermates and have a high perinatal lethality rate. *Igf1*^{-/-} mice that survive the prenatal period have severe growth retardation and are infertile. These data suggest that IGF-I plays a role in both prenatal and postnatal growth and development (9,10). Knockout of the *igf2* gene in mice results in severe fetal growth retardation; the animals are smaller at birth (60% of normal size) than *igf1*^{-/-} mice (9) (10). Ablation of the *igf1r* gene results in more profound prenatal growth retardation than disruption of either *igf1* or *igf2* alone because both IGF-I and IGF-II signal through *igf1r* (11). Using Cre/lox technology, liver specific *igf1* knockout mice were developed (11). These mice have a 75% reduction in serum IGF-I levels with little impairment of growth, indicating that local production of IGF-I regulates body growth.

Transgenic mice with generalized IGF-I overexpression have contributed to our understanding of IGF action in development and growth (12). Tissue-specific overexpression of IGF-I in mice has been used to study the local actions of IGFs. Zhao et al generated transgenic mice with osteocalcin promoter driven IGF-I to examine the effects of locally produced IGF-I in bone (13). These mice show increased matrix apposition rate at 3 weeks of age and greater bone volume and density at 6 weeks of age, at which time peak bone mass is achieved in the mouse. There were no differences in cortical bone width at 3- and 6-weeks of age and no differences in static or dynamic bone histomorphometric parameters at 24 weeks; there were also no differences in calvarial width and porosity. However, there was an increase in bone formation in the absence of

increase in osteoblast number, suggesting that the primary effect of IGF-I is to increase osteoblast function.

Jiang et al developed pOBCol3.6-IGF transgenic mice expressing IGF- I in bone driven by a 3.6 kb fragment of the type I collagen (Col1a1) promoter (14). This study showed that calvariae from pOBCol3.6-IGF mice had increased rates of collagen synthesis and cell replication in vitro, and increased calvarial width and histomorphometric resorption parameters in vivo. The phenotype was dependent on transgene dosage. They concluded that IGF-I can increase bone turnover. Osteoblast cultures from pOBCol3.6-IGF mice provide a unique model to examine the autocrine/paracrine effects of IGF-I on proliferation and differentiation

GENERAL OBJECTIVE

To examine the autocrine/paracrine effects of IGF-I on the proliferation and differentiation of cells of the osteoblast lineage using primary calvarial cells from pOBCol3.6-IGF-I transgenic mice.

- **Hypothesis:** Transgenic overexpression of IGF-I in osteoblasts stimulates proliferation and osteogenic differentiation

SPECIFIC OBJECTIVES

1. To examine primary osteoblast cultures established from pOBCol3.6-IGF-I mice for cell growth by measuring cell number over time.
2. To examine primary calvarial osteoblast cultures established from transgenic pOBCol3.6-IGF mice for the expression of differentiation markers by Northern blot analysis and reverse transcriptase-polymerase chain reaction (RT-PCR), including OC, BSP, Col1a1, AP and the formation of mineralized nodules.
3. To examine primary osteoblast cultures established from pOBCol3.6-IGF-I/Col2.3-IGF mice for Col2.3-GFP expression, a late marker of osteoblast differentiation. GFP fluorescence will be analyzed by fluorimaging whole cultures.

MATERIALS AND METHODS

Transgenic mice

Mice carrying the pOBCol3.6-IGF transgene were previously generated by embryo microinjection in the Transgenic Animal Facility (now called the Gene Targeting and Transgenic Facility) at the University of Connecticut Health Center. The transgene contains 3.6 kb of the rat Col1a1 promoter, a small segment of the rat Col1a1 first intron, most of the rat Col1a1 first exon, the murine IGF-I cDNA and the bovine growth hormone polyadenylation site (14). A FLAG epitope was placed between the signal peptide and the coding region of IGF-I (Figure 1A). Transgenic (TG) lines were established by breeding the founder lines with CD-1 mice. TG line 99-324-7 was used for these experiments. Hemizygous TG male mice were bred with wild type (WT) female CD-1 mice. Roughly half of the resulting progeny were hemizygous TG, the other half WT. Genotypes of the offspring were determined by PCR of tail DNA. All animal work was performed in compliance with the guiding principles in the “Care and Use of Animals” in the American Journal of Physiology, using protocols approved by the Animal Care and Use Committees of the University of Connecticut Health Center, Farmington, CT (protocol number 2004-18). For most experiments, hemizygous pOBCol3.6-IGF mice were crossed with homozygous Col2.3-GFP mice to generate pOBCol3.6-IGF (TG) and wild type (WT) progeny in a hemizygous Col2.3-GFP background. There was usually an equal representation of each genotype in the litters.

Genotyping by PCR

Mouse tail genomic DNA was extracted using the Wizard genomic DNA extraction kit (Promega, madison, WI, USA). PCR genotyping was carried out using primers corresponding to first intron of rat Collalgene (5'ACCCTCCTCCATTTTAGCC-3') and the FLAG sequence (5'CATCGTCGTCCTTGTAGTC-3'), which generates a unique 700 bp transgene product. DNA from WT mice does not give a product (Figure 1B). PCR cycles were 94°C, 30 sec; 65°C, 30 sec; 72°C, 2 min. The cycle number was 32. PCR products were fractionated by electrophoresis on a 1% agarose gel in 1X TBE buffer and visualized by ethidium bromide staining.

All the experiments were done in primary cell cultures. For each individual experiment independent cell digestions were performed. For cell growth experiments four wells per group were analysed. For AP staining, AP activity and von Kossa staining experiments, three wells per group were analysed. For Northern blot analysis and GFP expression studies, one sample per group was analysed. All experiments were repeated at least once and in most cases twice.

Primary osteoblast cultures

Calvarial osteoblasts were obtained from 6- to 8-day-old neonatal calvariae from WT and TG mice. During this time, the presence of the pOBCol3.6-IGF transgene in offspring was determined by PCR of tail DNA. After genotyping results were obtained, hemicalvariae of the same genotype were grouped and subjected to four sequential 15-

min digestions at 37°C on a rocking platform with an enzyme mixture consisting of 1.5 U/ml collagenase P in PBS and 0.05% trypsin/1mM EDTA. The first digest was discarded. The second to fourth digests were pooled. The cell pellet was collected after centrifugation at 2000 rpm for 10 min. Cells were resuspended in DMEM and passed through a 40 µm cell strainer. Cell numbers were counted using Coulter Counter (Coulter Corporation, Miami, USA). Cells were plated at an initial density of 15000 cells/cm² per well in 6-well culture dishes (Costar, Corning, NY) in DMEM containing 10% FBS and P/S. Medium was changed after 24 h and again on day 4. Osteogenic differentiation agents, 50 µg/ml ascorbic acid and 4 mM β-glycerophosphate, were added to the medium. Cultures were fed with medium containing fresh differentiating supplements every other day for the duration of the experiment.

RNA extraction and Northern blot analysis

RNA was extracted from primary calvarial osteoblast culture on days 7, 14, and 21 using the TRI^{ZOL} method. A 0.8 ml aliquot of TRI^{ZOL} Reagent (Life Technologies, Grand Island, NY, USA) was added to each culture well. Cells were transferred to 15 ml polypropylene tubes (Falcon, Becton Dickinson, Franklin Lakes, NJ) and immediately homogenized with a Polytron (PowerGen 700, Fisher Scientific) for 30 sec. Chloroform was added to tubes to give a final concentration of 1:5 (vol/vol). Tubes were mixed on a Vortex for 15 sec, and phases were allowed to separate for 5 sec. After centrifugation at 9,000 rpm for 20 min at 4°C, the upper phase was transferred to a fresh polypropylene tube. After isopropanol precipitation, the pellet was re-dissolved in 300 µl of GTC buffer (4.5 M guanidinium isothiocyanate, 10 mM β-mercaptoethanol, 15 mM sodium N-lauryl

sarcosine and 10 mM sodium citrate, pH 7.0) followed by precipitation with 300 μ l isopropanol. The precipitate was washed once with 80% ethanol, drained and re-dissolved in 50 μ l of diethylpyrocarbonate (DEPC) treated water.

For Northern blot analysis, 12-20 μ g of RNA from each group was denatured, loaded onto a 1% agarose gel with 6% formaldehyde and size fractionated by electrophoresis in MOPS buffer. RNA was transferred onto a Gene-Screen plus Hybridization Transfer membrane (PerkinElmer Life Sciences, Inc. Boston, MA) by capillary pressure. RNA was cross-linked to each membrane by UV irradiation. Prehybridization of each membrane was performed in 1X Pre-Hyb solution (Molecular Research Center, Cincinnati, OH) at 42°C for at least 3 h, followed by hybridization overnight with 32 P-labeled cDNA probes in a high efficiency hybridization system (Molecular Research Center, Cincinnati, OH) at 42°C with 5-6 million cpm/ml for each probe. The following cDNA probes were used: rat Coll1a1, mouse BSP, mouse OC and chick β -actin. Probes were labeled using the random primer method using [32 P]dGTP (3000 Ci/mmol, New England Nuclear, Boston, MA). Membranes were washed and exposed to a phosphorimager. The intensity of the radioactive signal was quantified using ImageQuant. Kodak BioMax MR- photographic film (Sigma Chemical Co., St. Louis, MO) was placed over the membrane with an enhancing screen for exposure at -80°C, and developed using a Kodak developer. The signal obtained with each cDNA was normalized to the signal obtained by hybridization with a radiolabeled β -actin probe.

RT-PCR for transgene expression

RNA (3 µg) was reversed transcribed into cDNA in the presence of oligo (dT) primers, RNase inhibitor and M-MLV reverse transcriptase at 42°C. After heating at 80°C for 10 min, PCR was performed using the RT product and transgene specific primers corresponding to the Col1a1 first exon (5'-AGCAGACGGGAGTTTCACCTC-3') and the FLAG epitope (5'-CATCGTCGTCC TTGTAGTC, which produce a 400 bp band. PCR was carried out with a Perkin Elmer GeneAmp system at 94°C for 30 sec, 65°C for 30°sec, and 72°C for 2 min for 25 cycles. Amplified products were fractionated by electrophoresis on a 5% polyacrylamide gel and visualized by ethidium bromide staining. The transgene band was normalized with murine glyceraldehyde 3-phosphate-dehydrogenase (G3PDH), which was assessed using the RT product and a primer pair that amplifies a 983 bp band.

AP staining and activity

AP expression in cell cultures was determined by histochemical staining and a quantitative activity assay. For AP staining, cells were rinsed twice with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde. An AP substrate mixture (AP staining kit, Sigma Diagnostic, Inc., St. Louis, MO) was added and the plates were incubated at room temperature, while protected from light, for 15 min for color development.

For AP activity assays, cell layers were removed from the culture plates in scrapping buffer (0.04M Tris, 1 mM EDTA, 0.15 M NaCl); cell pellets were collected after a brief centrifugation. Cells were lysed in Tris buffer (10 mM Tris, 0.15% Triton x-100) and subjected to 3 free-thaw cycles. After centrifuge at 14,000 rpm for 5 min, supernatant was collected. A 20 μ l aliquot of supernatant from each sample was added to each well in duplicates in a 96-well plate (Costar, Corning, NY), and incubated with an assay mixture of p-nitrophenyl phosphate. Plates were scanned for spectrophotometric analysis using a plate reader (μ Quant plate reader, Bio-Tek, Winooski, VT). Absorbance is measured at 410 nm every 5 minutes for 30 min. Activity was calculated using KC junior software (Bio-Tek, Winooski, VT). AP activity was expressed as nmol/min/mg.

Von Kossa staining

Mineralization of the cells was assessed by the von Kossa method. Cells were fixed for 10 min in 2% paraformaldehyde in 0.1 M cacodylic buffer. The plates were incubated with 5% silver nitrate solution for 30 min under a bright light, washed with water, treated with a 5% sodium thiosulfate solution for 2-3 min, followed by washing with water and air drying. The von Kossa positive area of each well was quantitated by threshold segmentation using Metamorph image processing software (Center for Biomedical Imaging Technology, UCHC).

Fluorescence microscopy

GFP fluorescence in cell culture was visualized using an Olympus IX50 inverted microscope equipped with an IX-FLA inverted reflected light fluorescence (Olympus

America, Inc, Melville, NY). A specific excitation wavelength was obtained using filters for GFPtpz (exciter: D500/20; dichroic: 525DCLP; emitter: D550/40) and recorded with a SPOT-camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Fluorescent images were taken with equal exposure times for all groups. Phase contrast bright-field images were converted to grayscale. Magnifications of 4X, 10X and 20X were used. Bright-field/fluorescent composite images are generated using Adobe Photoshop software. The distribution and quantification of GFP fluorescence in cell culture was performed with FluorImager SI (Molecular Dynamics, Sunnyvale, CA, USA) using a 515-nm emission spectrum at PMT settings of 800. Images were processed with Image QuaNT software (Molecular Dynamics) and quantified by threshold segmentation using Metamorph image processing software (Center for Biomedical Imaging Technology, UCHC).

Statistics

The comparison of means between WT and TG groups was determined with the unpaired t-test. A value of $p < 0.05$ was considered to be a significant difference between groups.

RESULTS

pOBCol3.6-IGF transgenic mice were generated by Jiang et al as described previously (14). Transgenic mice were identified by genotyping DNA from tail biopsies and performing PCR using transgene specific primers. Hemicalvaria from 6-8 day old neonatal mice were extracted and pooled based on genotyping results. Primary cells were prepared by serial enzymatic digestion and cell proliferation and differentiation was assessed by various methods.

Cell proliferation in WT and TG primary osteoblast cultures

Primary osteoblasts were obtained by sequential enzymatic digestion of calvariae from 6-8 day- TG and WT mice. Cells were plated at an initial density of 40,000 cells/cm² (day 0) in 12-well culture plates and cell number per well was determined daily on days 1-7. A total of five experiments were done. Based on the cell number of cells plated on day 0, cell number was reduced by 20 percent in WT and by 40 percent in TG cultures on day 1 of culture. This indicated that the actual number of cells plating down was 60-80%. Cell growth in both groups slowed between days 3 and 4 (Figure 2A). At each day, there was only a small difference in cell number between WT and TG groups. However, the daily percentage increase in cell number was greater in TG cultures at days 4 and 5 (Figure 2B).

Osteoblast differentiation in WT and TG primary osteoblast cultures

Osteoblast differentiation was determined by measuring AP activity, von Kossa staining, GFP expression and osteoblast mRNA markers by Northern blot analysis.

AP activity

At days 14 and 21, AP staining was performed on three wells per group and AP activity was measured in three wells per group in a total of three experiments (Figure 3A). There was no noticeable difference between AP staining of WT and TG cultures. AP activity was measured as an early differentiation marker on days 14 and 21. No significant difference was seen in AP activity between TG and WT cultures at day 14. A 50% decrease in AP activity was seen in TG cultures at day 21 (Figure 3B).

Von Kossa staining

Mineralization was assessed by von Kossa staining at day 21. Von Kossa staining was assessed as a late differentiation marker in the same wells as AP staining in two experiments (Figure 4A). The von Kossa positive area of each well was quantitated by image analysis. There was no difference in von Kossa staining between WT and TG cultures (Figure 4B).

GFP expression

pOBCol2.3-GFP expression was used as a visual marker for late stage differentiation. GFP fluorescence was observed using fluorescent microscopy (Figure 5A) and quantified by Fluorimaging using Image QuANT software, followed by threshold segmentation using Metamorph image processing software (Figure 5B). Expression was seen on day 14 in both groups. GFP was strongly expressed in bone nodules within the cultures. Similar patterns and intensities of GFP were seen in WT and TG cultures. When the data from three experiments were pooled, there was no difference in pOBCol2.3-GFP expression in WT and TG cultures (Figure 5C).

mRNA markers

The expression of osteoblast differentiation markers including Colla1, BSP, and OC was determined by Northern blot analysis in three experiments (Figure 6A). All hybridization signals were normalized to actin. On day 7, OC and BSP mRNAs were not expressed in either WT or TG cultures. On day 7, Colla1 was expressed in both WT and TG cultures. There was a trend ($0.5 < p < 0.1$) towards lower Colla1 expression in TG cultures. On days 14 and 21, Colla1, BSP, and OC were expressed in both WT and TG cultures (Figures 6B and C). The relative expression of Colla1 in TG cultures increased between days 7 and 14 (Figure 6D). There was no difference in expression of these markers between WT and TG cultures.

Transgene expression

To measure transgene expression through out the culture period, transgene specific primers were used for RT-PCR analysis. The primers, corresponding to the Col1a1 exon and FLAG epitope (Figure 7A), generated a unique ~400 bp product with TG RNA. Cells from WT mice did not express the transgene (Figure 7B). In cells from TG mice, transgene mRNA was detected as early as day 7 and appeared to increase through out the culture period. The transgene band was normalized with murine glyceraldehyde 3-phosphate-dehydrogenase (G3PDH), which was assessed using the RT product and a primer pair that amplifies a 983 bp band

DISCUSSION

Bone remodeling is regulated by many hormones and growth factors including IGF-I. Understanding the cellular and molecular mechanisms and action of these factors will provide a better understanding of their role in normal and pathological bone remodeling. It is well known that IGF-I plays an essential role in normal growth and development. IGF-I exerts an anabolic action on protein and carbohydrate metabolism and regulates cellular activities involving cell proliferation, differentiation, and apoptosis (15).

To gain insight into the role of locally-produced IGF-I in bone, Jiang et al developed a transgenic mouse model with osteoblast-directed overexpression of IGF-I (pOBCol3.6-IGF). This model showed evidence of increased bone turnover (14). Calvariae were wider and increased porosity. Calvarial organ cultures from TG mice showed increased rates of collagen synthesis and thymidine incorporation, a measure of cell replication.

To understand the cellular and molecular mechanisms underlying the bone phenotype of pOBCol3.6-IGF mice, we examined osteoblast differentiation and bone cell proliferation in ex vivo cultures of primary osteoblasts established from TG and WT calvariae. We reasoned that the IGF-I transgene would become activated during the primary culture and have an affect on proliferation and/or osteoblast differentiation.

Primary calvarial cell cultures contain precursor cells for osteoblasts. They can be induced to undergo osteogenic differentiation and express osteoblastic markers *in vitro* in the presence of serum, ascorbic acid and β -glycerophosphate. These cells express multiple growth factors and their receptors including IGF-IR. The phenotype of primary calvarial osteoblasts may reflect their behavior *in vivo*. Therefore, studying the proliferation and differentiation in wild type and pOBCol3.6-IGF primary calvarial osteoblasts may provide insight into the mechanisms by which IGF-I regulates bone turnover *in vivo*.

We found that cell growth was higher in TG cultures compared to WT cultures. This finding was consistent with the finding in previous studies showing that IGF-I increase cell growth and proliferation (15). The reduced cell number one day after plating probably indicated that all of the plated cells did not adhere to the dishes. By day 5 and 6, cells reached confluence. The increase in cell growth could be attributed to either an increase in cell proliferation or a decrease in apoptosis. These were not measured in the present study.

AP was used as an early marker of osteoblast differentiation. Other early markers are Colla1 and activated leucocyte cell adhesion molecule (ALCAM). Compared to WT cells, there was a significant decrease in AP activity in TG cells on day 21 but not at day 14, indicating that late osteoblast differentiation was affected. However, the expression of other osteoblast markers including Colla1, BSP and OC, von Kossa staining and GFP expression were not altered in TG cultures. Thus, the inhibitory effect on osteoblast

differentiation was relatively modest and limited to a decrease in AP expression. However, in addition to IGF-I, neonatal mouse calvarial cells also produce other growth factors such as IGF-II, TGF β , and PDGF (16). Therefore, the interplay of multiple growth factor pathways is probably required for osteoblast proliferation and differentiation. It remains to be determined whether the lack of effect of the IGF-I transgene on osteoblast differentiation was the result of an increase in cell growth or the in vitro environment.

The increased expression of IGF-I in transgene bone could have produced compensatory changes in other components of the IGF-I pathway including expression of IGFBPs and IGF receptors (17, 18). For example, if the transgene led to an increase in IGFBP expression, this could mitigate the effect of transgenic IGF-I.

It has been demonstrated that IGF-I stimulates the proliferation of pre-osteoblasts to increase the pool of osteogenic progenitors, and promotes the progression of pre-osteoblasts to full osteoblast differentiation (19). However, the exact mechanisms through which IGF-I regulates osteoblast lineage progression remain unclear. Some investigators proposed that the effects of IGF-I are not mediated via targeting osteoprogenitors, but are largely restricted to more mature osteoblasts to maintain their differentiation (20-22). Results from our study demonstrate that IGF-I may affect proliferation rather than differentiation of primary calvarial osteoblasts because more prominent effects were observed on cell growth.

Based on this data, we conclude that IGF-I overexpression driven by the 3.6-kb *Col1a1* promoter fragment in primary calvarial osteoblasts from transgenic mice showed an increase in osteoblast proliferation but had no significant effect on differentiation.

SUMMARY AND CONCLUSIONS

The acquisition and maintenance of bone mass are complex processes regulated by systemic hormones and local growth factors. IGF-I plays an essential role growth, skeletal development and bone mass acquisition. IGF-I acts in both an endocrine manner, as it is produced and secreted by liver, and in a paracrine/autocrine manner, as it is produced by osteoblasts, to exert growth hormone-dependent and growth hormone-independent effects on bone.

A tremendous amount of knowledge has been gained regarding the *in vivo* actions of IGF-I with the development of transgenic and mutant animal models. To understand the cellular and molecular mechanisms underlying the bone phenotype of pOBCol3.6-IGF mice, we examined osteoblast differentiation and bone cell proliferation in ex vivo cultures of primary calvarial osteoblasts established from TG and WT calvariae. We found that cell growth was higher in TG cultures compared to WT cultures. A significant decrease in AP activity in TG cells was noted on day 21 indicating that late osteoblast differentiation was affected. Expression of other osteoblast markers including Colla1, BSP and OC, von Kossa staining and GFP expression were not altered in TG cultures. The inhibitory effect on osteoblast differentiation was relatively modest and limited to the decrease in AP expression. Thus, results from our study demonstrate that IGF-I may affect proliferation rather than differentiation of primary calvarial osteoblasts.

The increase in cell growth could have been due to increased cell proliferation or decreased apoptosis. A further study measuring cell proliferation (thymidine and Brdu

incorporation) and apoptosis (TUNEL assay and caspase activity) would give clear insight into the mechanism for increased cell growth in TG cultures.

Another possibility in the pOBCol3.6-IGF model could have been compensatory changes in other components of the IGF-I pathway including expression of IGFBPs and IGF receptors. A logical extension of our work would be to examine the expression of IGFBPs in cultured osteoblasts including IGFBPs 2 and 4.

Figure 1. Schematic diagram of the pOBCol3.6-IGF transgene and PCR genotyping of mice

A) The pOBCol3.6-IGF transgene contains a rat Col1a1 gene fragment from -3518 to +1594 bp, including 3.6 kb of Col1a1 promoter sequence and part of the Col1a1 first exon (Col3.6) and most of the Col1a1 first intron, murine IGF-I cDNA (IGF-I) in which the FLAG (FL) epitope was inserted between the signal peptide (SP) and the mature coding region of IGF-I and the bovine growth hormone polyadenylation sequence (pA). PCR genotyping was carried out using primers corresponding to regions of the Col1a1 first intron and the FLAG epitope as shown by the arrows.

B) A typical PCR genotyping analysis of progeny generated by breeding a hemizygous pOBCol3.6-IGF transgenic (Tg) mouse with a wild-type (Wt) mouse. DNA was extracted from tail biopsies and amplified by PCR using the as described in the Methods and Materials. DNA from Tg animals gives a unique 700 bp transgene product. M, ØX molecular weight size markers. Lanes labeled P and B indicate a positive control and a water blank, respectively. The remaining lanes indicate the genotyping results for individual progeny, which were found to be Tg or Wt.

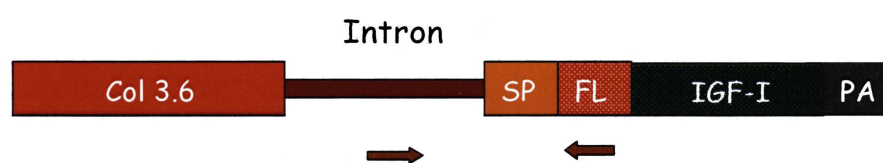
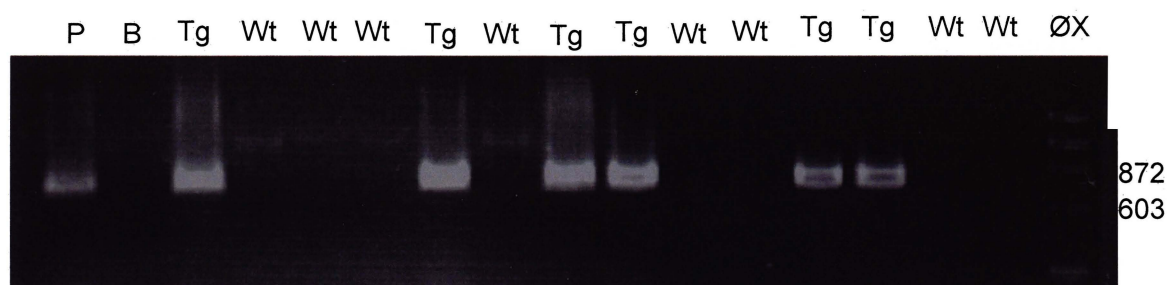
A**B**

Figure 2. Cell growth in primary calvarial osteoblast cultures.

A) Cell growth in wild type (Wt) and transgenic (Tg) cultures from day 1 to day 6. The graph is representative of five independent experiments.

B) Percent cell growth during defined intervals in Wt and Tg cultures from day 1 to day 6. At each day, cell number in Wt cultures was set to 1.0 and the ratio of cell number in Tg compared to Wt cultures was calculated. Each value is the mean \pm SEM of five experiments. The graph shows results pooled from five independent experiments.

*Different from Wt, $p < 0.05$.

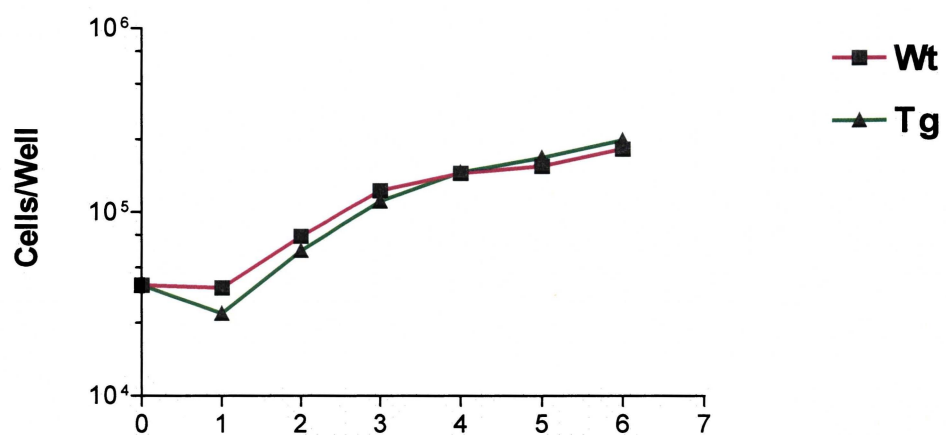
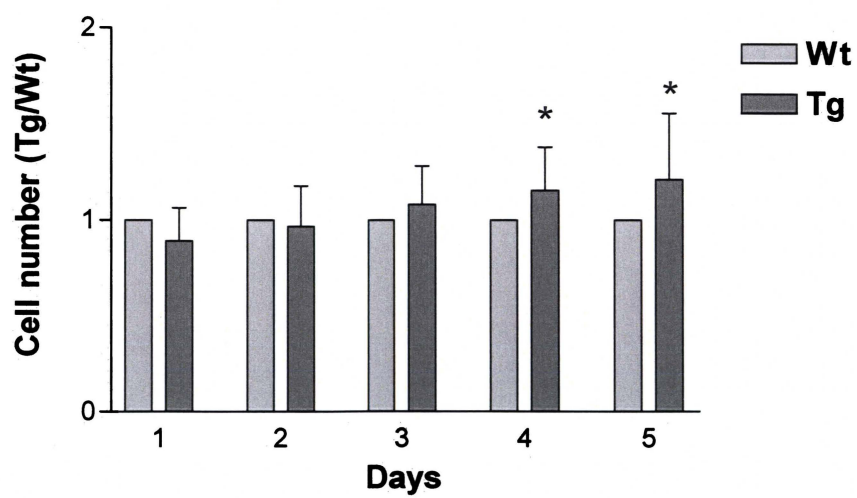
A**B**

Figure 3. Alkaline phosphatase staining in primary osteoblast cultures.

A) Alkaline phosphatase (AP) staining of wild type (Wt) and transgenic (Tg) cultures on day 21. The white areas in the cultures represent mineralization.

B) AP activity in Wt and Tg cultures on days 14 and 21. Each value is the mean \pm SEM of three experiments. For each experiment AP activity in Wt cultures was set to 1.0 and the ratio of AP staining in Tg compared to Wt cultures was calculated. Data shown were pooled from three independent experiments. *Different from Wt, $p < 0.05$.

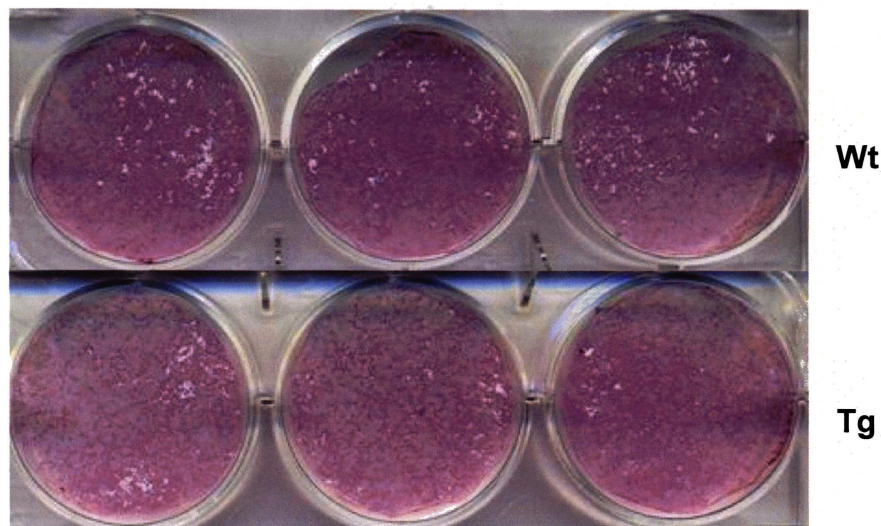
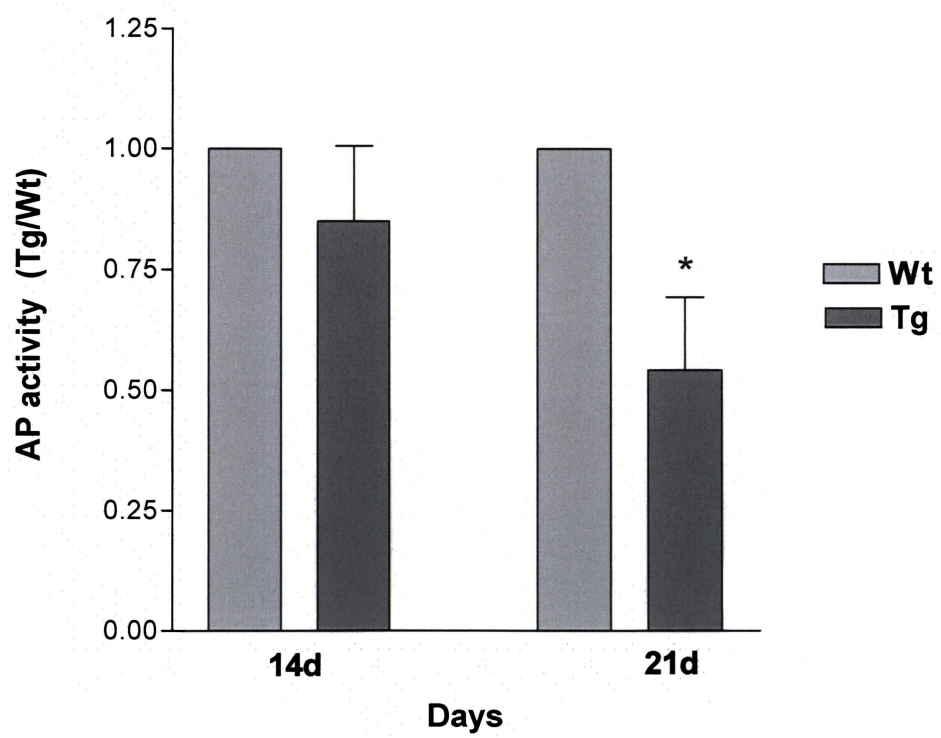
A**B**

Figure 4. Mineralization in primary calvarial osteoblast cultures.

A) Following alkaline phosphatase (AP) staining, day 21 wild type (Wt) and transgenic (Tg) cultures were stained with Von Kossa to assess mineralization. The black color represents mineralization.

B) Image analysis to quantitate mineralization. The von Kossa stained areas on day 21 Wt and Tg cultures were imaged as described in Materials and Methods. Von Kossa stained area in Wt cultures was set to 1.0 and the ratio of von Kossa staining in Tg compared to Wt cultures was calculated. Each value is the mean \pm SEM of four cultures. Data shown were pooled from three independent experiments.

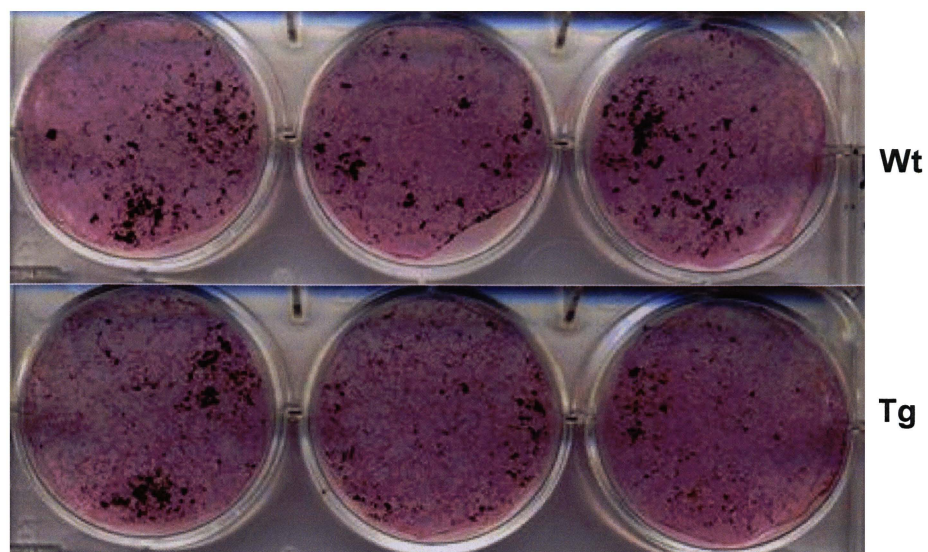
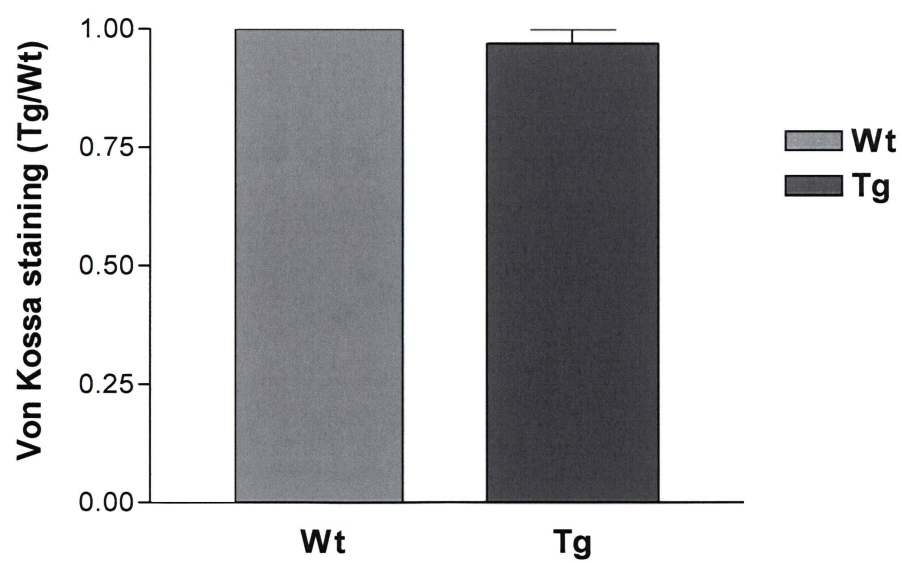
A**B**

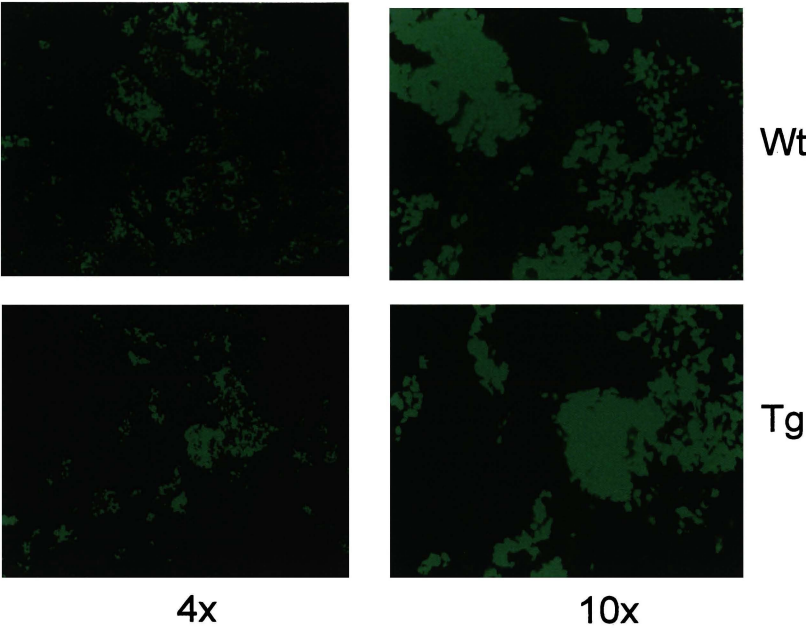
Figure 5. GFP expression in primary calvarial osteoblast cultures.

A) Hemizygous pOBCol3.6-IGF mice were breed with homozygous pOBCol2.3-GFP mice to generate progeny that were transgenic (Tg) or wild type (Wt) with respect to the pOBCol3.6-IGF transgene in a hemizygous pOBCol2.3-GFP background. GFP fluorescence in Wt and Tg cultures is shown at two magnifications (4x and 10x).

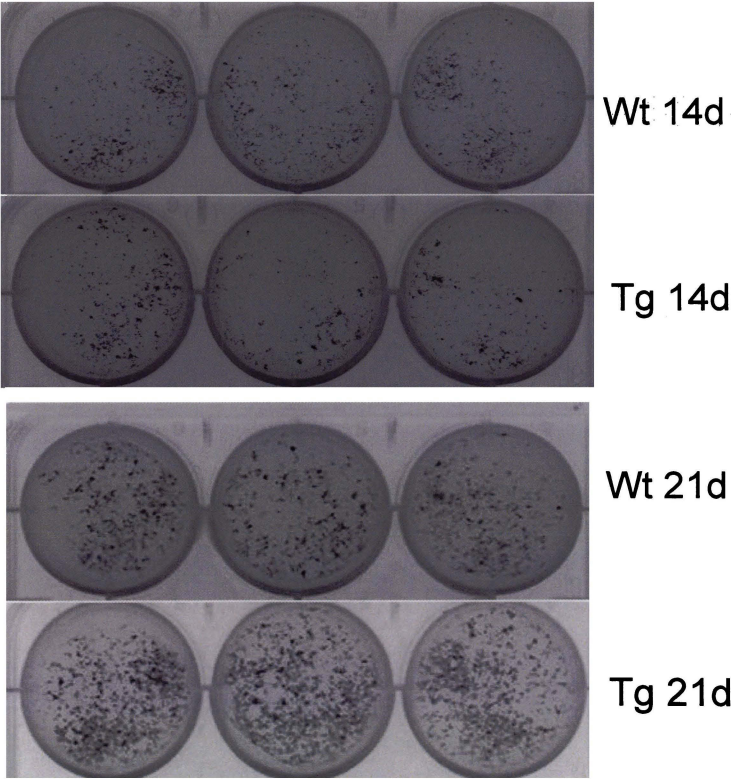
B) Fluorimager images of GFP expression in day 14 and 21 Wt and Tg cultures.

C) Quantification of GFP fluorescence by fluorimaging on day 21 Wt and Tg cultures. GFP fluorescence in Wt cultures was set to 1.0 and the ratio of GFP fluorescence in Tg compared to Wt cultures was calculated. Each value is the mean \pm SEM of six cultures. Data shown were pooled from three independent experiments.

A



B



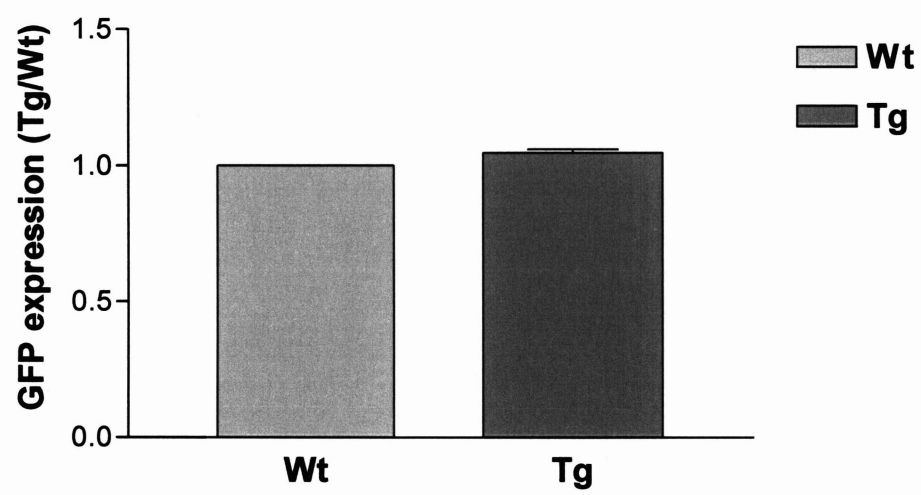
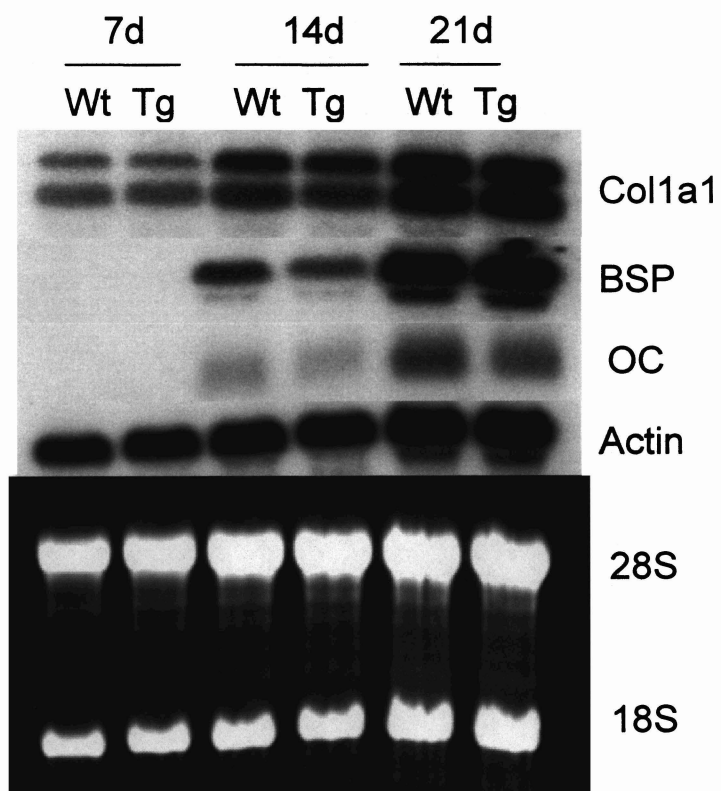
C

Figure 6. Expression of mRNA markers in primary osteoblast cultures.

on days 7, 14, and 21 as assessed by Northern blot analysis. Data shown are representative of three independent experiments.

A) Northern blot gel. RNA was extracted from wild type (Wt) and transgenic (Tg) cultures and subjected to Northern blot analysis as described in Material and Methods. The gel was probed with *Colla1*, BSP, OC and actin cDNAs (top). Ethidium bromide staining of 28S and 18S ribosomal RNA is shown (bottom). For each Northern blot experiment, the intensity of the bands were quantitated using a phosphorimager and the ratio of mRNA expression to actin was indicated. This value in Wt cultures was set to 1.0 and the ratio of gene expression in Tg compared to Wt cultures was calculated. Each value is the mean \pm SEM. Data shown were pooled from three independent experiments.

B) Quantitation of OC mRNA expression. C) Quantitation of BSP mRNA. D) Quantitation of *Colla1* mRNA expression.

A

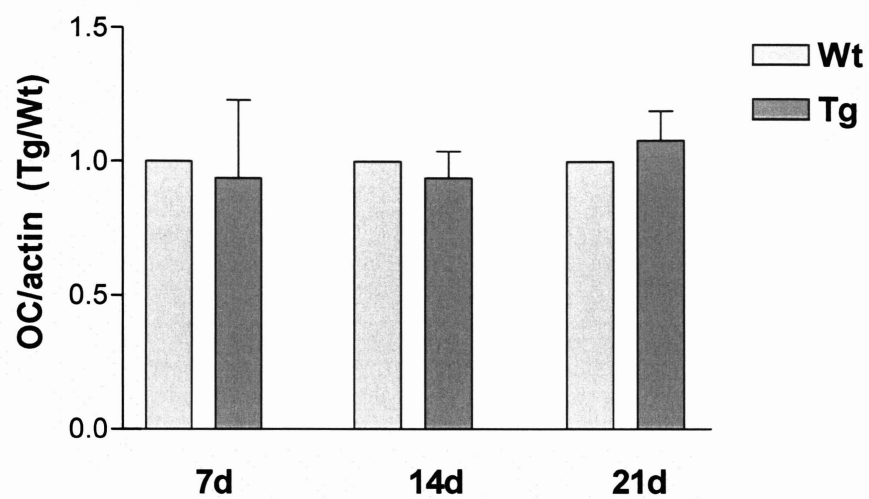
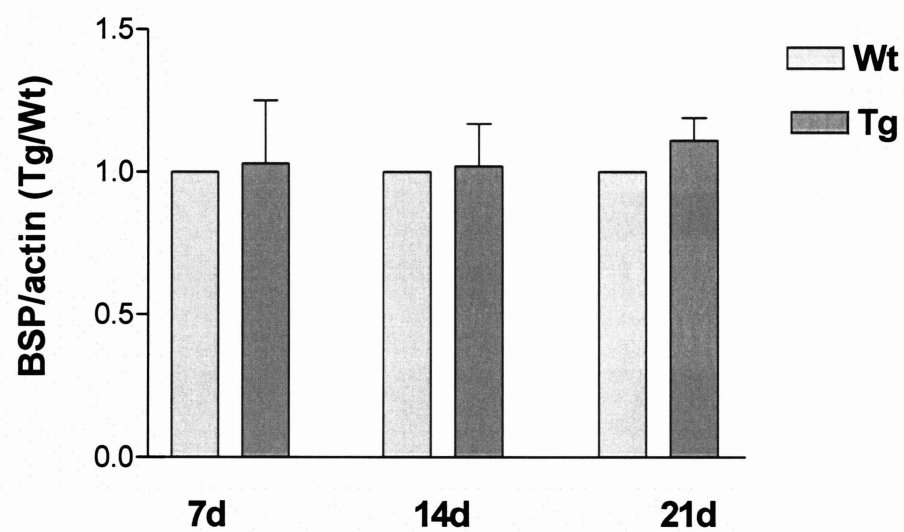
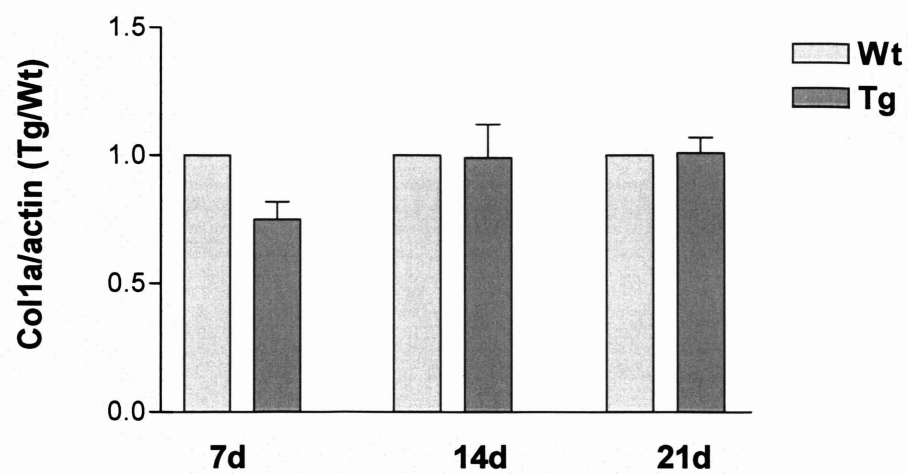
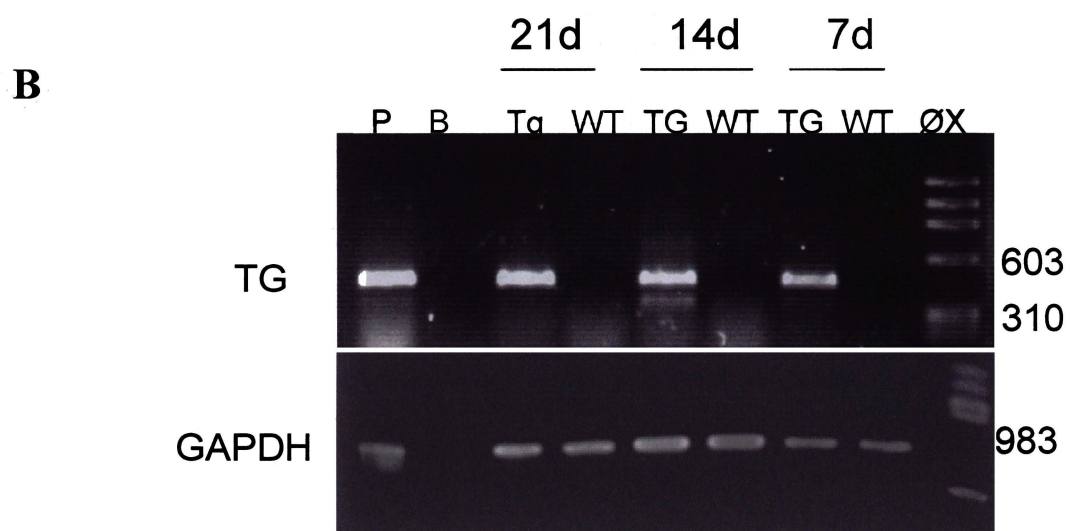
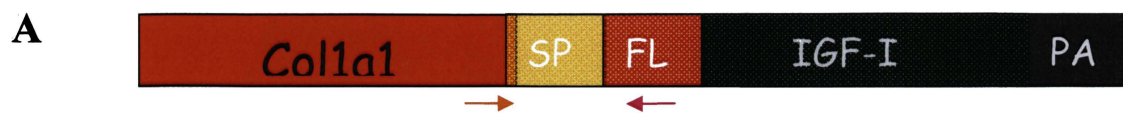
B**C****D**

Figure 7. Expression of pOBCol3.6-IGF transgene mRNA expression by RT-PCR analysis.

RNA (3 µg) from day 7, 14 and 21 wild type (Wt) and transgenic (Tg) cultures was used in a RT-PCR analysis with primers corresponding to the rat Col1a1 first exon and the FLAG epitope. In Tg cultures, this analysis produced a 400 bp product. P, positive control; B, water blank; ϕ X, molecular weight markers. RNA integrity was verified by amplification with G3PDH primers.



LITERATURE CITED

1. **Liu J EAF** 1996 The osteoblast lineage. In: Bilezikian JPR, Lawrence G; Rodan, Gideon A. (ed) Principles of bone biology, 1 ed. Academic Press, Inc., San Diego, pp 51-58
2. **Gary S. Stein JBL, Janet L.Stein, Andre J. Van Winjen, Baruch Frenkel, and Martin Montecino** 1996 Mechanisms Regulating Osteoblast Proliferation And differentiation. In: Bilezikian JPR, Lawrence G; Rodan, Gideon A. (ed) Principles of Bone Biology, 1 ed. Academic Press, Inc, San Diego, pp 70-72
3. **Zapf J, Schmid C, Froesch ER** 1984 Biological and immunological properties of insulin-like growth factors (IGF) I and II. Clin Endocrinol Metab 13:3-30
4. **Hock JM, Centrella M, Canalis E** 1988 Insulin-like growth factor I has independent effects on bone matrix formation and cell replication. Endocrinology 122:254-60
5. **Delany AM, Rydziel S, Canalis E** 1996 Autocrine down-regulation of collagenase-3 in rat bone cell cultures by insulin-like growth factors. Endocrinology 137:4665-70
6. **Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC** 1998 Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest 102:274-82
7. **Gohel A, McCarthy MB, Gronowicz G** 1999 Estrogen prevents glucocorticoid-induced apoptosis in osteoblasts in vivo and in vitro. Endocrinology 140:5339-47

8. **Daughaday WH, Hall K, Raben MS, Salmon WD, Jr., van den Brande JL, van Wyk JJ** 1972 Somatomedin: proposed designation for sulphation factor. *Nature* 235:107
9. **Powell-Braxton L, Hollingshead P, Warburton C, et al.** 1993 IGF-I is required for normal embryonic growth in mice. *Genes Dev* 7:2609-17
10. **Baker J, Liu JP, Robertson EJ, Efstratiadis A** 1993 Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 75:73-82
11. **Yakar S, Liu JL, Le Roith D** 2000 The growth hormone/insulin-like growth factor-I system: implications for organ growth and development. *Pediatr Nephrol* 14:544-9
12. **Mathews LS, Hammer RE, Brinster RL, Palmiter RD** 1988 Expression of insulin-like growth factor I in transgenic mice with elevated levels of growth hormone is correlated with growth. *Endocrinology* 123:433-7
13. **Zhao G, Monier-Faugere MC, Langub MC, et al.** 2000 Targeted overexpression of insulin-like growth factor I to osteoblasts of transgenic mice: increased trabecular bone volume without increased osteoblast proliferation. *Endocrinology* 141:2674-82
14. **Jiang J** 2001 Development and Phenotypic Characterization of Transgenic Mice with Bone-directed Overexpression of Insulin-like Growth Factor-1. Thesis. University of Connecticut, Farmington
15. 1996 principles of bone biology, 1 ed. Academic Press, san Diego

16. **Slootweg MC, van Buul-Offers SC, Hoogerbrugge CM, et al.** 1990
Characterization of growth factor activity produced by fetal mouse osteoblasts. *J Endocrinol* 124:301-9
17. **Mohan S, Strong DD, Lempert UG, Tremollieres F, Wergedal JE, Baylink DJ** 1992 Studies on regulation of insulin-like growth factor binding protein (IGFBP)-3 and IGFBP-4 production in human bone cells. *Acta Endocrinol (Copenh)* 127:555-64
18. **Gabbitas B, Canalis E** 1995 Bone morphogenetic protein-2 inhibits the synthesis of insulin-like growth factor-binding protein-5 in bone cell cultures. *Endocrinology* 136:2397-403
19. **Chihara K, Sugimoto T** 1997 The action of GH/IGF-I/IGFBP in osteoblasts and osteoclasts. *Horm Res* 48 Suppl 5:45-9
20. **Thomas T, Gori F, Spelsberg TC, Khosla S, Riggs BL, Conover CA** 1999 Response of bipotential human marrow stromal cells to insulin-like growth factors: effect on binding protein production, proliferation, and commitment to osteoblasts and adipocytes. *Endocrinology* 140:5036-44
21. **Walsh S, Jefferiss CM, Stewart K, Beresford JN** 2003 IGF-I does not affect the proliferation or early osteogenic differentiation of human marrow stromal cells. *Bone* 33:80-9
22. **Langdahl BL, Kassem M, Moller MK, Eriksen EF** 1998 The effects of IGF-I and IGF-II on proliferation and differentiation of human osteoblasts and interactions with growth hormone. *Eur J Clin Invest* 28:176-83